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ORIGINAL PAPER

Breaking seed dormancy in *Cupressus atlantica* Gaussen, an endemic and threatened coniferous tree in Morocco

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Abstract: *Cupressus atlantica* Gaussen (Cupressaceae) is an endemic and endangered coniferous tree geographically restricted to the N'Fis valley in South-Western Morocco. Like many forest species, *C. atlantica* exhibits dormancy which delays and reduces germination. To improve seed germination, different pre-treatments were conducted on *C. atlantica* seeds after storage for different periods (one, two and five years) including: scarification with sandy paper; soaking seeds in hot distilled water at 60°C and 80°C for 15 min and soaking seeds for 48 h in a gibberellic acid (GA3) at 1,000 and 2,000 mg·L^{-1}. Results showed that scarification with sandy paper increased the germination rate of Atlas cypress by up to 67%, indicating that the species possess essentially an exogenous dormancy (physical dormancy) due to the hard seed coat (hardseededness). Exogenous application of gibberellic acid (GA3) at 1,000 mg·L^{-1} was also effective in breaking seed dormancy and germination induction. These two treatments induced faster speed germination expressed by low number of days to first germination (8−10 days) and low values of mean germination times (MGT). However, germination rate, under any treatment, is greatly dependent on the year of seed collection. Seeds collected in year 2004 gave the highest value, suggesting that even after five years of storage, the germination capacity of *C. atlantica* seeds could remain high. This observation is very interesting in the *ex-situ* conservation of such endemic and endangered species where the production of seeds is irregular over the years.

Keywords: *Cupressus atlantica*; endemic; endangered; breaking seed dormancy; germination capacity; hand scarification; *ex-situ* conservation

Introduction

Germination is a critical stage for the establishment of plant species, especially those that grow naturally in arid and semi-arid environments (Alouani and Bani Aameur 2004). Several factors may influence germination percentages in natural environments. Among these factors, seed dormancy contributes in part to germination failure of many species under adverse environmental conditions (Bewley 1997). There are two categories of dormancy: coat-enhanced dormancy (seed coat dormancy) where the embryos isolated from these seeds are not dormant and embryo-dormancy (internal dormancy) where the embryos themselves are dormant (Bewley 1997; Ellery and Chapman 2000). Seed dormancy is reported to be an adaptive mechanism that ensures the survival of the species through periods of environmental stress (Gutterman 1993; Bell et al. 1995; Baskin and Baskin 1998; Gusano et al. 2004; Kermode 2005). Many unpredictable factors can control the intensity of this dormancy and lead to high degrees of intra-specific variation at several levels (Andersson and Milberg 1998). The environmental conditions of the maternal plants during seed maturation are reported to be an important factor in controlling seed dormancy and germination, accounting for a significant proportion of the variation in percent germination and likely responsible for the differences in germination between seeds from different years (Andersson and Milberg 1998).

Cupressus atlantica Gaussen, a Cupressaceae, commonly known as Atlas cypress, is an endemic coniferous tree, restricted

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to the N'Fis valley in the High Atlas Mountains of Morocco. The species and its natural geographical distribution is represented by several stands and a number of isolated trees distributed over a total area of 200 km^2 (Bechir 2004; Alifriqui et al. 1996). The species is drought and frost-resistant and tolerates a variety of soils. It grows at 1,100–2,200 m elevation in a Mediterranean climate with rainfall of 350–700 mm/year. Maximum summer temperature reaches 30°C and winter minimums below 0°C (Alifriqui et al. 1996). *C. atlantica* is threatened by habitat degradation, overgrazing, and limited natural regeneration. Indeed, the species has been classified by Food and Agriculture Organization (FAO) among the 17 world forest species whose genetic inheritance is most impoverished (FAO 1976). Aghbar station, considered as one of the most important *C. atlantica* forests. There, the total area occupied by *C. atlantica*, has declined from 5,500 ha to 1,460 ha, over a 50-year period (1933-1983) (Ech-Chamikh 1983; Barbero et al. 1990). To maintain and improve these Atlas cypress forests, the Moroccan Forest Service manages many nurseries to produce seedlings under controlled conditions. Even in the nursery, seed germination has been poor, perhaps in part because of dormancy problems. The improvement of the efficacy and efficiency of restoration efforts requires a better understanding of the germination requirements of this species. There is little available information on the type of seed dormancy in *C. atlantica,* the effect of seed breaking treatments on dormancy release and germination, nor the effect of year of harvest / length of storage periods. It's recognized that the germination capacity of many forest species varies over a period of time depending on the age of the seed (or period of storage) and environmental conditions (Reyes and Casal 2001).

The objectives of the present study were to determine treatments for Atlas cypress seeds that can improve germination and which are adaptable to various nursery situations. Additionally, we assessed the effect of storage duration on germination rates. The effect of physical, mechanical and chemical pre-sowing treatments on the germination response of *C. atlantica* seeds, after storage for different periods of time, were examined.

Materials and methods

Seed collection

Cones of *C. atlantica* were collected in September of years 2004, 2007 and 2008 from the Aghbar population, a site of biological and ecological interest and retained by forest administration as part of a natural seed bank, often used in reforestation program using C. *atlantica*. It is interesting to note that during the years 2005 and 2006, the production of cones was very low in the Aghbar population, thus those years were not included in this study. Once the cones were collected they were exposed to sun for 24 h, to stimulate natural opening, after which the seeds were collected. Seeds were stored until 2009, under controlled conditions of temperature (4°C) and humidity (about 30%). When the germination tests were carried out the seeds had been in storage for between one and five years. In autumn 2009, seeds were exposed to different treatments believed to break their dormancy and enhance germination. Before treatment, seeds were surface sterilized for 20 min in 3% sodium hypochlorite, rinsed in distilled water and dried before the experiment.

Seed breaking treatments

Seeds were submitted to mechanical, physical and chemical treatments. The mechanical treatments consisted of vigorously rubbing the seeds with 100 grit sandpaper to abrade the seed coat. Physical treatments involved soaking the seeds in hot distilled water at 60°C and 80°C for 15 min. The chemical treatment consisted of soaking seeds for 48 h in a gibberellic acid (GA3) solution at 1,000 or 2,000 $mg \cdot L^{-1}$. After treatments, seeds were placed on a single Whatman (N° 3) filter paper in 9-cm Petri dishes moistened with 5 ml of distilled water. Four replicates of 30 randomly selected seeds were used for each treatment. For each experiment, a batch of untreated seeds was used as control. The dishes were placed in a thermostatically controlled incubator (± 1°C) in the dark at 20°C, conditions which have been cited as the optimum temperature for seed germination of the species (Bechir 2004; Ouahmane 2007). Placement of the dishes was randomized.

Dishes were checked every two days and for a period of 28 days (incubation period). Germinated seeds (young radicles over 5 mm in length) were counted and removed from each Petri dish. Final germination percentage and mean germination time (MGT) were calculated, the latter according to the following formula (Scott et al. 1984):

$$\text{MGT (days)} = \sum (n_i \times d_i)/N$$

where n_i is the number of germinated seeds on day i, d the number of days after beginning of the experiment, and N the total number of seeds germinated. If no seeds germinated, MGT was assumed to be 28 days to permit analysis of variance.

Additionally, cross-sections of untreated seeds were observed with a JEOL JSM-5500 Scanning Electron Microscope (SEM) and micrographs were recorded on 55 positive/negative Polaroid film. Seed coat surfaces were coated with a carbon layer using an SPI-Module Carbon Coater.

Statistical analysis

The data were subjected to an analysis of variance (ANOVA) test. Two-way factorial ANOVA and Student-Neuman-Keuls (SNK) multiple comparison tests were used. Germination percentages were arcsine-transformed using the SPSS 10.0 software package before analysis.

Results

This study clearly showed that all specific treatments, as well as storage duration, affected the percent germination and mean

germination time of *C. atlantica* seeds. The influence of these factors is significant when considered in isolation or when the interaction between them is taken into account (Table 1). Non-treated seeds of *C. atlantica* germinated to a maximum of only 28% (Table 2). The highest germination percentage was observed for seeds that had been hand scarified with sand paper (Table 2). The mean final germination for hand scarification was over 67%, indicating that this was the most efficient treatment. Exogenous application of gibberellic acid (GA3) at 1,000 mg·L^{-1} was also effective in increasing the germination rate of *C. atlantica* seeds (Table 2), although the percentage of germination was not as high as for hand scarification treatment. Mechanical scarification and GA3 treatments induced a faster rate of germination (lower number of days to the first sign of germination (8–10 days), and low values of mean germination times (MGT) when compared to other treatments (Fig. 1, Table 2).

Table 1. **Effects of treatment and years of collection on the final germination percentage and mean germination time of *Cupressus atlantica* seeds**.

Source of variance	df	F value	P level
Final germination percentage			
Treatment	5	72.304	0.000
Year	2	102.786	0.000
Treatment*Year	10	11.922	0.000
Error	54		
Total	72		
Mean germination time			
Treatment	5	59.543	0.000
Year	2	34.169	0.000
Treatment*Year	10	7.595	0.000
Error	54		
Total	72		

Table 2. Final germination rate (GR) and mean germination time (MGT) of *Cupressus atlantica* seeds under different treatments

Treatments	Year of collection					
	2004		2007		2008	
	Germination rate (%)	Mean germination times (Days)	Germination rate (%)	Mean germination times (Days)	Germination rate (%)	Mean germination times (Days)
Control	28.33 (16.88)**ef**	24.44 (3.08)**ABC**	3.66 (1.92)**g**	18 (0.10)**AB**	24.16 (8.76)**f**	26.55 (1.46)**A**
H_2O (60°C)	84.16 (15)**bc**	19.20 (1.92)**C**	3.33 (2.72)**g**	24 (3,46)**A**	13.33 (9.81)**f**	26.25 (0.96)**A**
H_2O (80°C)	88.33 (8.81)**b**	19.92 (1.21)**C**	59.16 (8.33)**de**	15,55 (0,87)**D**	52.50 (10.67)**de**	22.96 (0.90)**ABC**
Mechanical scarification	98.66 (1.63)**a**	15.02 (1.14)**D**	76.66 (9.18)**cd**	15,32 (1,09)**D**	67.53 (8.76)**d**	14.64 (2.07)**D**
GA_3 (1000 mg·L^{-1})	85 (7.93)**bc**	15.48 (0.62)**D**	61.66 (6.38)**d**	11,16 (0,77)**D**	60 (11.22)**d**	19.08 (3.75)**BC**
GA_3 (2000 mg·L^{-1})	83.33 (7.2)**bc**	15.04 (0.78)**D**	50.83 (11.01)**de**	11,8 (2,00)**D**	47.5 (13.71)**de**	20.65 (1.53)**BC**

Values of each parameter having the same letter are not significantly different at 5%.

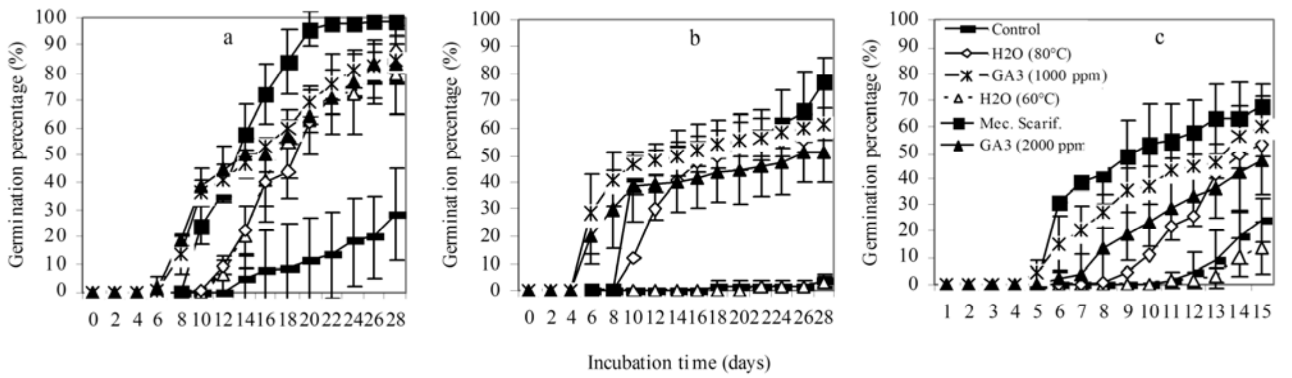

Fig. 1 Cumulative germination rate (mean ± SE) of *Cupressus atlantica* seeds collected from Aghbar station during the three years (2004 (a), 2007 (b) and 2008 (c)).

The analysis of germination of *C. atlantica* revealed that germination rates differ between seeds collected in different years (and therefore stored for different lengths of time). The highest germination rate was recorded in the oldest seeds, collected in 2004. The lowest values were observed in the youngest seeds, from 2008 (Table 2). The effect of the hot water treatment was strongly influenced by the age of the seeds. The treatment in hot water (60°C) yielded poor germination seeds collected in 2007 and 2008 (>14%), whereas, for seeds collected in 2004 the germination percentage was higher (84.16%). An increase in the temperature of water used for the treatment, from 60°C to 80°C, gradually and significantly increased the rate of germination of *C.*

atlantica seeds. In fact, seeds soaked in hot water at 80°C gave the best result with germination percentage exceeding 50% for seeds collected in 2007 and 2008, whereas, for seeds collected in 2004, the percentage of germination averaged 88% (Table 2).

SEM did not reveal any significant difference in structure nor thickness of the palisade layer of the seed coats collected in different years (Fig. 2).

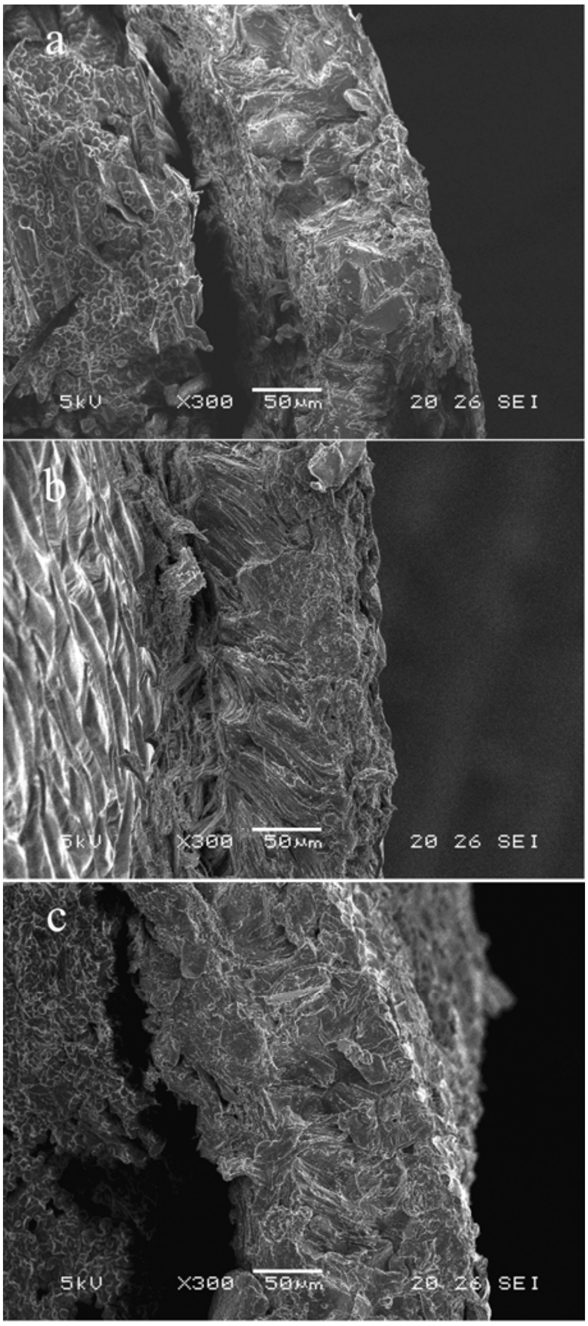

Fig. 2 Cross section of the seed coat of *Cupressus atlantica* Gaussen (a: 2004; b: 2007; c: 2008).

Discussion

This study suggests that the hard seed coats of *C. atlantica* seeds lead to exogenous dormancy (physical dormancy), and that this is one of the main inhibitor of germination. Removing the thick cellular layer beneath the seed coat by hand scarification with abrasive paper attained almost full germination. Hand scarification has been reported by several authors as the best treatment to overcome seed coat impermeability of many forest tree species (Mapongmetsem et al. 1999; Valbuena and Tarrega 1998). The positive effect of gibberellic acid treatment seen in this study is likely in part due to the induction of certain enzymes that are involved in the cell walls weakness of seed coat of many species (Leubner-Metzger 2002; 2005; Nakajima et al. 2004).

Variation in germination between years of seed production is frequently reported and has been attributed to several factors including environmental conditions under which plants are grown (Vleeshouwers et al. 1995; El-Keblawy and Al-Ansari 2000; El-Kebalwy and Al-Rawai 2006). Maternal environment can alter germination by affecting chemical composition, seed provisioning (e.g. mineral, photosynthetic and phytohormone resources) (Baskin and Baskin 1998; Galloway 2002) and structure and thickness of the seed coat (Lacey et al. 1997; Luzuriaga et al. 2006). However, in this study SEM did not revealed a significant difference in structure and thickness of the palisade layer of the seed coat between seeds collected in different years (Fig. 2). This suggests that the variability seen in this study was probably attributed to the physiological state of embryos due to year-to-year variation in climatic conditions during seed development. Indeed, it was reported that seed germination rate can be greatly affected by humidity levels during seed maturation (Philippi 1993), and that levels of dormancy are higher in drier environments than in wet ones (Qaderi and Cavers 2000). Rainfall in Aghbar region during seed maturation (February to September) for the three years of collection was lower in 2007 and 2008 than in 2004 (Fig. 3). Variation in dormancy between years has been suggested to be an adaptation to unpredictable environments (Andersson and Milberg 1998). Beckstead et al. (1996) suggested that populations from more favorable but somewhat unpredictable environments are expected to show more variation between years in germination than those from less variable environments.

The results obtained in this study could also suggest a positive effect of storage period on improved seed germination, leading to the observed difference between seeds collected in different years. The data showed that the old seeds (2004) germinated to higher levels, compared to younger ones (2007 and 2008). The same observations were reported in other species such as *Eucalyptus globulus* (Reyes and Casal 2001). It appears that embryos may require longer storage periods to reach full development. In contrast, some authors reported that several conifers exhibited good long storability, but the prolonged storage period induced seed deterioration and reduced the seed quality (Terskikh et al. 2008). Further studies will be necessary in order to elucidate the

effect of long term storage on seed viability and quality in this species.

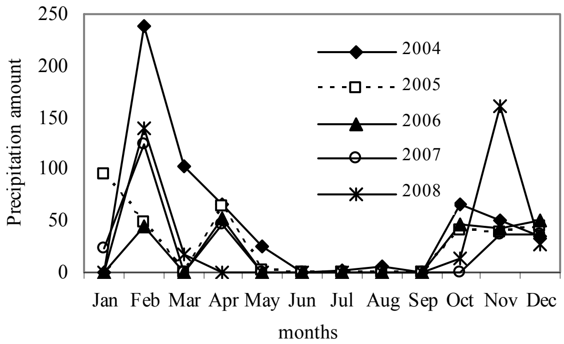

Fig. 3 Monthly variation of precipitations amount during years 2004 to 2008 in Aghbar station.

Conclusions

From this investigation, it can be concluded that the very poor natural regeneration of *C. atlantica* is in part attributed to physical dormancy caused by a hard seed coat. Hand scarification appeared to be the most effective method to break this dormancy. However, the percentage of germination depended greatly on the year of seed collection or period of storage. This study also indicated that the germination capacity of *C. atlantica* seeds was high, even after five years of storage. The same observation was reported on some coniferous forest tree such as *Pinus halepensis* and *Pinus radiata* (Reyes and Casal 2001). These findings make a significant contribution to the conservation efforts for this endemic and endangered species with irregular annual seed production.

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